

Ets1 as a Target of MicroRNA-1 in Cutaneous Squamous Cell Carcinoma

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ABSTRACT

Cutaneous squamous cell carcinoma (SCC) is the second most common form of skin cancer in the United States. Using microarrays, we identified lower levels of *microRNA-1* (*miR-1*) expression in skin from mice that are susceptible to chemically-induced skin cancer, compared to skin from resistant mice. Murine SCC cell lines show reduced levels of *miR-1* compared to normal skin. We hypothesize that the decreased expression of *miR-1* in susceptible mice and SCC cell lines suggests a possible role of *miR-1* as a tumor suppressor in skin. To test our hypothesis, we transfected a *miR-1* precursor into a murine SCC cell line, A5. Upon transfection, we measured decreased cell proliferation and migration, and increased apoptosis. Using *in silico* prediction programs, we identified *Ets1* as a potential direct target of *miR-1*. We observed decreased *Ets1* mRNA and protein expression in A5 cells transfected with *miR-1*. To determine if *Ets1* is a direct target of *miR-1*, we co-transfected an *Ets1* 3'UTR luciferase construct and *miR-1* into a normal keratinocyte cell line, C5N. We observed decreased luciferase expression at 24 hours post-transfection, suggesting that *Ets1* is a direct target of *miR-1*. In conclusion, *miR-1* appears to target *Ets1* and act as a tumor suppressor by increasing apoptosis and decreasing proliferation and migration. In future studies, we will characterize the role of *Ets1* in SCC cell proliferation, apoptosis, and migration, and identify other targets and tumor-related phenotypes associated with *miR-1*.

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CHAPTER 1

INTRODUCTION

1.1. Cutaneous Squamous Cell Carcinoma (SCC)

As the largest organ in the human body, the skin serves a primary function to protect internal tissues and organs from detrimental environment effects. The skin consists of two layers, the dermis and epidermis. The dermis provides mechanical support whereas the purpose of the epidermis is largely protection. Five layers of cells are combined to form the epidermis, which include the following in order from deep to superficial: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum. Keratinocytes begin to differentiate in the basal layer and differentiate outward towards the surface of the skin. Cells fully differentiate upon reaching the stratum corneum [1].

Nonmelanoma skin cancer (NMSC) results from tumorigenesis of the epidermis and includes basal cell carcinoma (BCC) and squamous cell carcinomas (SCC). Rogers et al. estimates that in the United States, there are nearly 3,500,000 NMSCs and greater than 2,100,000 individuals treated each year. Further, recent studies show that around 1,000,000 new NMSCs arise in the U.S. each year, and there was a dramatic 76.9% increase in NMSC treatment procedures for the Medicare population between 1992 and 1996 [2]. Common treatment for NMSCs is surgical removal, and costs of actinic keratoses and NMSCs in the U.S. combined exceed \$3.5 billion each year [3].

Cutaneous squamous cell carcinoma (SCC) is the second most common form of skin cancer with approximately 700,000 diagnoses made each year [4]. Actinic keratosis, pre-cancerous keratinocytes *in situ*, commonly precedes the development of

SCC, and occurs on the outermost layer of the epidermis. SCC is caused by ultraviolet radiation exposure from the sun and most commonly occurs on the arms, legs, nose, and ears [5]. Other SCC risk factors include: old age, smoking, human papilloma virus, infection, and chronic inflammation of the skin. Also, men are at a higher risk of obtaining SCC than women. Tumors appear as raised, ulcerous, papules [6].

1.2. MicroRNAs

MicroRNAs are small, ~25 nucleotide strands of RNA that are thought to inhibit gene expression via translational inhibition [7]. The mechanism by which this occurs first begins with transcription of the 1-3 kb primary microRNA (pri-miR) by RNA polymerase II [8]. The pri-miR is then processed by the enzyme DROSHA to a 70 bp, stem-loop precursor microRNA (pre-miR) which is exported from the nucleus to the cytoplasm by Exportin-5/RanGTP complex [9,10]. In the cytoplasm, the pre-miR is cleaved by DICER to the mature, ~22 nucleotide, microRNA (miR). The miR then complexes with the RISC protein complex and binds to a complimentary seed region (7-9 bp) in the 3' untranslated region (3'UTR) of a target gene mRNA where it will cause degradation if it is exactly complimentary or otherwise induce translational repression through steric hindrance [11]. However, recent studies suggest that microRNAs may also bind to the 5'UTR, other microRNAs, promoter regions, and ribonucleoproteins [12]. MiRs have been shown to act as tumor suppressors or oncogenes, and are thought to regulate about 30% of the human genome [12,13,14,15].

1.3 *MicroRNA-1 (miR-1)*

MicroRNA-1 (miR-1) was identified as a cardiac and skeletal muscle specific microRNA (myomiR) that falls into a family of six other miRs [16,17,18,19]. It has been shown to help regulate skeletal muscle and cardiomyocyte progenitor cell proliferation and differentiation [20,21,22]. *MiR-1* has also been shown to be pro-apoptotic in skeletal muscle and cardiomyocytes [23,24]. Further, *miR-1* is down-regulated in human lung, liver, and skeletal muscle cancer where decreased cell growth and proliferation is observed upon introduction of *miR-1* into the cancer cell lines [25,26,27].

In previous studies performed by other members of the Toland lab, a microarray revealed decreased *microRNA-1 (miR-1)* expression in a mouse strain susceptible to chemically induced [12-dimethylbenz(a)anthracene (DMBA) and 2-O-tetradecanoyl-phorbol-13-acetate (TPA) induced] SCC compared to a mouse strain that is resistant [28]. These results were further confirmed via quantitative real-time PCR indicating a 2-fold decrease of *miR-1* expression in the skin of susceptible mice compared to resistant mice. Additionally, the A5 SCC cell line shows greatly reduced (undetectable) levels of *miR-1* when compared to the C5N normal keratinocyte cell line. The inverse correlation of expression in mice susceptible to cutaneous SCC compared to those resistant suggests that *miR-1* may play a role in tumorigenesis.

1.4 *Ets1*

Ets1 is a transcription factor oncogene shown to be involved in regulation of the tumorigenesis promoting EGFR-Ras-MEK1/2 MAPK pathway [29,30,31,32]. *Ets1* is

also known to fuse with the E26 avian leukaemia virus *c-myb* proto-oncogene which is also an important causation of tumorigenesis [33,34].

Specifically, *Ets1* has been shown to play a role in SCC tumor development and progression [6,35,36]. *Ets1* acts as a SCC tumor promoting gene by inducing SCC cell proliferation in transgenic mice over-expressing *Ets1* [35]. Studies show that *Ets1* regulates genes important in apoptosis, angiogenesis, migration, invasion, and specifically, matrix metalloproteinases which enhance cell migration [36,37,38]. In addition, it is over-expressed in human SCC malignancies [37,39,40,41,42,43].

1.5 Study Rationale and Hypothesis

There are three parts to this study.

1. To test the role of *miR-1* as a tumor suppressor gene
2. To determine if *Ets1* is a direct target of *miR-1* as well as the specific phenotypes associated with *Ets1*.
3. To determine the mechanism of *miR-1* down-regulation in SCC.

By definition, a gene is considered a tumor suppressor if its loss or inactivation results in neoplastic growth irregularities [44]. When present at normal expression levels, tumor suppressor genes help regulate normal cell processes, but when inactive a cell can experience phenotypes such as uncontrollable proliferation, decreased apoptosis, de-regulated cell cycles, increased migration, increased invasion, or increased metastasis. These phenotypes lead to tumorigenesis. The decreased expression of *miR-1* in mice susceptible to cutaneous squamous cell carcinoma compared to resistant mice, alongside data from previous studies supporting a role of

miR-1 in important tumor phenotypes (e.g. proliferation, apoptosis, and migration) led to the hypothesis. The hypothesis of this thesis is that *miR-1* may act as a tumor suppressor in cutaneous squamous cell carcinoma by down-regulating *Ets1*.

Our *in silico* studies show that *Ets1* is a predicted target of *miR-1*. *Ets1* is also implicated as an oncogene. Confirmation of the means by which *miR-1* regulates *Ets1* expression provides us with a better understanding of the mechanism by which *miR-1* acts as a SCC tumor suppressor. Down-regulation of *Ets1* could occur by *miR-1* binding to the *Ets1* 3'UTR and inhibiting translation. (We assume that the RISC proteins subsequently form a complex with the microRNA when the microRNA binds to the 3'UTR.) However, down-regulation of *Ets1* expression via *miR-1* could also occur if *miR-1* down-regulates a gene responsible for up-regulation of *Ets1*, such as a transcription factor. Experimentation was necessary to differentiate between these previous two possible mechanisms of regulation. A specific step of the mechanism could be exploited by future studies to investigate and possibly develop potential SCC therapeutics. We hypothesize that *miR-1* acts as an SCC tumor suppressor by directly reducing expression of the transcription factor oncogene, *Ets1*, via binding to the *Ets1* 3'UTR.

According to *in silico* prediction programs, www.microRNA.org and www.targetscan.org, there are three binding sites for *miR-1* on the *Ets1* 3'UTR. The accepted mode of microRNA regulation of target genes in various cancers is via direct binding of the microRNA to the 3'UTR of the target gene. Studies have shown that even 1-2 microRNA binding sites on the 3'UTRs of target genes is sufficient for down-regulation, which suggests three binding sites may be more than sufficient in our study

[25,41,45]. However, we wanted to confirm that *miR-1* is directly binding to the 3'UTR rather than indirectly regulating another intermediate that can bind to the 3'UTR, such as another microRNA.

Finally, differential expression of microRNAs in tumor versus normal tissue is often associated with epigenetic alterations such as DNA methylation or chromatin silencing [26,46,47,48,49,50,51]. Specifically, *miR-1* expression has been shown to be suppressed in human liver and lung cancer via histone-silencing, and its expression was rescued upon treatment of cancer cells with a histone-deacetylase inhibitor, trichostatin A (TSA). *MiR-1* has also been shown to be suppressed in human liver cancer by *Methylation*, and its expression was rescued upon treatment of the cells with a de-*Methylating* agent, 5-Azacytidine (5-AzaC). This leads us to investigate these forms of *miR-1* down-regulation in cutaneous SCC.

CHAPTER 2

MATERIALS AND *METHODS*

2.1 Cell Lines

Our experiments are performed using murine cell lines. In particular, we used the A5 SCC cell line and C5N normal keratinocyte cell line. The A5 cell line was directly derived from a mouse SCC tumor, and the C5N cell line was directly derived from mouse normal skin. Both cell lines are immortalized. Cells were grown in Dulbecco's modifications of Eagle's medium (DMEM, Cellgro) supplemented with 10% fetal bovine serum (GIBCO, Invitrogen) and 5% penicillin streptomycin (Invitrogen). Cells were trypsinized with 0.05% trypsin and 0.5 mM EDTA in HBSS with sodium bicarbonate, calcium, and magnesium.

2.2 Transfections

All transfections were performed using Lipofectamine 2000. The transfections for measuring proliferation, apoptosis, migration, and *miR-1* target gene expression were performed according to the Lipofectamine 2000 protocol in a final volume of 13 mL of OPTI-MEM in a 10 cm dish (Invitrogen). Co-transfections were performed per manufacturer's recommended protocol as the other transfections, with the co-transfected oligonucleotides transfected together with no adjustments in volumes as specified by the original transfection protocol. 130,000 A5 cells were plated one day prior to transfection in a 10 cm dish. Cells were transfected at 50% confluency. For each dish, 600 pmol of *miR-1* precursor molecule (Ambion), 600 pmol of scrambled precursor negative control (Ambion), and 500 ng of pCMV *Ets1* construct lacking the *Ets1* 3'UTR

(donated as a gift from the laboratory of Michael Ostrowski) were transfected into A5 cells. For brevity, the *miR-1* precursor molecule, scrambled negative control precursor microRNA molecule, and *Ets1* pCMV construct lacking the 3' UTR will be referred to as *miR-1*, negative control, and *Ets1* respectively throughout the rest of manuscript.

2.3 Cell Proliferation

One day post-transfection with either a scrambled precursor negative control or precursor to *miR-1*, A5 cells in the 10 cm dish were trypsinized, and 2,000 cells were replated in quadruplicate into a 96 well dish. Proliferation was measured at 24, 48, and 72 hours post-transfection using the MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Cell Proliferation Kit I according to manufacturer's instructions (Roche). The cells were solubilized four hours after the addition of the MTT reagent using the kit's provided solubilization reagent. The solution was then incubated for 24 hours, and absorbance was then measured at 550 nm using a spectrophotometer.

2.4 Apoptosis

One day post-transfection with a scrambled precursor negative control or a precursor to *miR-1*, A5 cells in the 10 cm dish were trypsinized, and 2,000 cells were replated in triplicate into a 96 well dish. Apoptosis was measured at 24, 48, and 72 hours post-transfection using a Caspase-Glo 3/7 Assay according to manufacturer's instructions (Promega). Samples were incubated with the caspase substrate for 1 hour. Caspase 3/7 activity was then measured using a luminometer.

2.5 Cell Migration

At 60 hours post-transfection, A5 cells transfected in a 10 cm dish with a scrambled precursor miR negative control or precursor to *miR-1* were trypsinized, and 30,000 cells were replated in triplicate into the upper chamber of the 8.0 μ m-translucent Transwell (Greiner Bio One). Prior to plating the cells, the Transwell was coated with fibronectin (5 ng/ μ L). Epidermal growth factor (10 ng/mL) was added to the lower chamber. Cells migrated for 12 hours after which they were fixed in 1% gluteraldehyde and stained with crystal violet. Cells were solubilized with Sorenson's buffer, after which absorbance was measured at 595 nm using a spectrophotometer.

2.6 Target Genes

Predicted targets of *miR-1* were identified using the *in silico* prediction programs, www.microRNA.org and www.targetscan.org. Predicted targets are identified based on the degree of complimentary alignment between *microRNA-1* and the 3'UTR binding site on the target gene's mRNA. The greater the degree of complimentary alignment, the higher score a target receives, and then the more likely it is that the gene may be a predicted target. From the list of predicted targets provided, specific genes were chosen to focus on based on alignment scores and literature review describing the function these genes have been shown to play in cellular processes. After selecting the predicted targets, their mRNA and protein expression was measured post *miR-1* transfection using SYBR Green quantitative real-time PCR and western blotting respectively.

2.7 *Ets1* 3'UTR Luciferase Reporter Assay

A murine *Ets1* 3'UTR insert containing three predicted *miR-1* binding sites was cloned into a pGL3 *Firefly* luciferase reporter vector (Luc-*Ets1* 3'UTR Promega). Two base pair substitutions were generated in each *miR-1* binding site in the *Ets1* 3'UTR using site-directed mutagenesis (Luc-*Ets1* 3'UTR-M, Stratagene). Mutated binding sites were generated to avoid creation of new microRNA binding sites as verified by www.patrocles.org.

C5N normal keratinocyte cells were plated in triplicate into a 12-well dish at 24 hours prior to transfection. At 70% confluency, 0.10 ug Luc-*Ets1* 3'UTR and Luc-*Ets1* 3'UTR-M were each co-transfected with 10 pmol of a scrambled precursor negative control miR or *miR-1* into each well. 0.10 ug of PRL-TK (TK-driven *Renilla* luciferase vector) was also transfected into each well. Transfections were performed using Lipofectamine 2000 according to protocol (Millipore). Luciferase expression was measured using a luminometer at 24 hours post-transfection and *Firefly* luciferase expression was normalized to *Renilla* expression.

2.8 De-methylation Studies

A5 SCC cells were plated in triplicate into a 12-well dish 24 hours prior to drug treatment. At 70% confluency, cells were treated with 5-azacytidine (5-AzaC; 5uM or 10 uM) for 48 hours, trichostatin A (TSA; 0.3 uM) for 24 hours, or a combination of 5-AzaC treatment for 48 hours and TSA treatment for 24 hours. RNA was harvested post-

transfection and *miR-1* expression was measured via Taqman quantitative real-time PCR.

2.9 Chromatin Immunoprecipitation

A chromatin immunoprecipitation assay was carried out on DNA/chromatin complexes isolated from A5 SCC and C5N normal keratinocyte cell lines according to protocol (Millipore). 130,000 cells were 70% confluent and cross-linked with formaldehyde (Immunoprecipitation was performed using the histone H3 dimethyl K9 antibody (Abcam; ab1220). The *miR-1* promoter region was amplified using *miR-1* promoter primers from Integrated DNA Technologies (Table 1). The amplified DNA was run on a 1% agarose gel with ethidium bromide, and the bands were visualized using UV radiation. DNA from a no-antibody control was used for normalization for loading control. Semi-quantification to measure pixel intensity was performed using Alpha Imager software.

2.10 Quantitative-PCR

RNA was extracted from 24, 48, and 72 hours transfections using RiboZol Extraction Reagent (AMRESO). MicroRNA cDNA was generated from the extracted RNA according to manufacturer's protocol (Applied Biosystems). Target gene cDNA was generated from extracted RNA using the I-Script kit according to manufacturer's protocol (BioRad). Transfection efficiency was verified via measuring *miR-1* and *Ets1* expression at 24, 48, and 72 hours post-transfection using quantitative real-time PCR

(qPCR). mRNA expression of potential *miR-1* target genes were also measured using qPCR.

Taqman qPCR was performed in triplicate according to manufacturer's protocol to measure *miR-1* expression (Applied Biosystems). *MiR-1* expression was presented as a percent relative expression to *sno202* expression using the ΔCT equation (equation: $(2^x) \times 100$ where $x = \text{control gene expression} - \text{target gene expression}$). Probes were obtained via Applied Biosystems.

SYBR green qPCR was performed in triplicate according to manufacturer's protocol and was used to measure *Ets1* and other target gene expression (Integrated Technologies). Primers were designed using Integrated DNA Technologies. *Ets1* and other potential *miR-1* targets were normalized to *L19* expression using the ΔCT equation (equation $(2^x) \times 100$ where $x = \text{control gene expression} - \text{target gene expression}$).

2.11 Western Blotting

Protein was extracted from 72 hours transfections via solubilization of the cells in RIPA buffer (50 mM Tris base pH 8, 150 mM NaCl, 1% NP40, 0.10% SDS) and subsequent removal of the supernatant (30 μ L). Equal amounts of protein (30 μ g each) were separated by 10% SDS-PAGE and then transferred to nitro-cellulose membrane. The membranes were blocked with a buffer containing 5% nonfat milk in phosphate-buffered saline with 0.05% Tween 20 for 30 min and incubated overnight with a 1:5000 dilution of *Ets1* primary antibody (provided as a gift by the laboratory of Michael Ostrowski). After 3 washes with phosphate-buffered saline with 0.05% Tween 20, the membranes were incubated with a 1:10,000 dilution of an anti-rabbit secondary

antibody for 2 hours α -tubulin (Santa Cruz Biotechnology) was used as a loading control.

2.12 Calculations and Statistical Analysis

Averages and standard deviations were determined for experimental and negative control variables using Microsoft Excel. A two-tailed student's t-test was used to determine significance. Differences between variables were considered significant if $p < 0.05$.

2.13 Primers: Table 1

Amplified DNA	Forward	Reverse
<i>miR-1</i> Promoter	5' CTG T CC AGG TAA GCC CTT TGA TTC 3'	5' GCA GAACAA TGC CAA CAG GGACA 3'
<i>Ets1</i>	5' TGTATG AGT GGA GCA GCA CTG TGT 3'	5' AGG TAG GGT CTC CAT TAA CCT 3'
<i>Met</i>	5' AAC GGG TAT TGG GAA GAC CCT GAA 3'	5' ATC CCG TCT AAC AGG AAG AAG GCT 3'
<i>Bag4</i>	5' ACT CCA CGG AAG TTC CAAACA CCT 3'	5' T TCCAG GGT TCT GTG AAG CAG GAT 3'
<i>Ptk9</i>	5' TTC CAG GCT TTG GAG AAG GT G AGT 3'	5' AGT CTC CTT CGT GGG AAT GCT TGT 3'
<i>Ets1</i> 3'UTR	5' GTACGT ACA CTA GTA TCC CTT CCACTG TAT AGC TGC CT 3'	5' GCA TGC ATC CGC GGC GCT ACA TCT CTA AGCTAC CTC AGT TC 3'

TABLE 1. Primer sets used for PCR amplification throughout experimentation. The *miR-1* promoter was amplified for ChIP experimentation. *Ets1*, *Met*, *Bag4*, and *Ptk9* were amplified in SYBR Green qPCR. The *Ets1* 3'UTR was amplified for a cloning insert.

RESULTS

3.1 *MiR-1* Reduces Cell Proliferation

To determine if *miR-1* affects cell proliferation, *miR-1* and a scrambled precursor miR negative control (NC) were transfected into A5 cells, and cell proliferation was measured at 0, 24, 48, and 72 hours post transfection via an MTT assay (Figure 1).

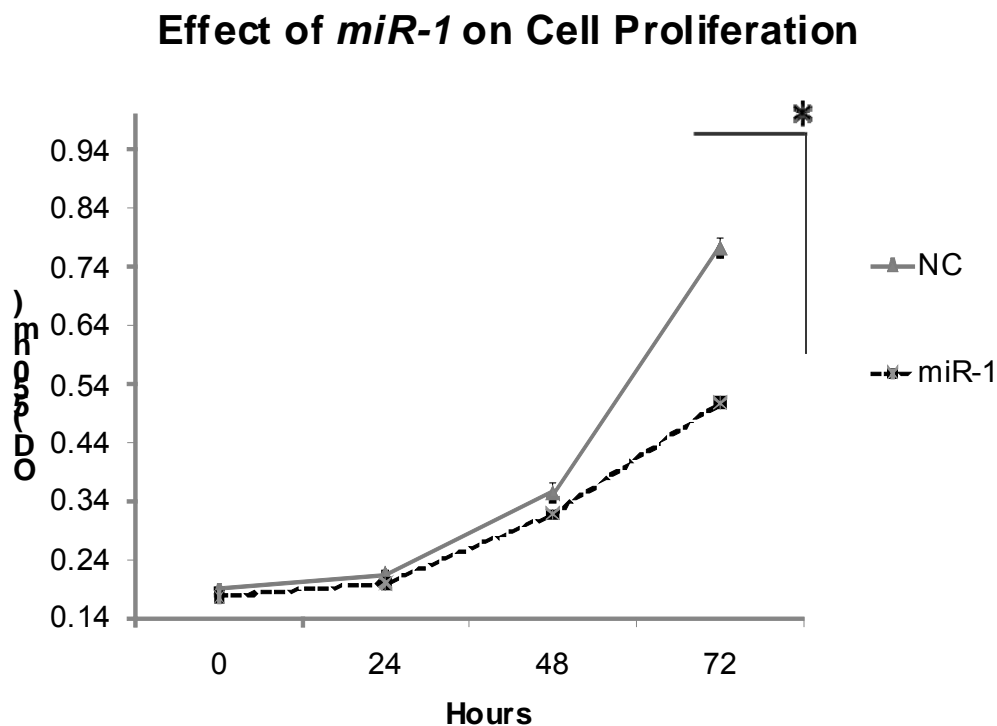


FIGURE 1. Cell proliferation (OD 550) was measured via an MTT assay at 0, 24, 48, and 72 hours post transfection in A5 cells transfected with *miR-1* (black, square) and scrambled precursor miR negative control (gray, triangle, NC). Proliferation at each time point was measured in quadruplicate. * $p < 0.0001$.

A significant decrease in cell number was observed at 72 hours post-transfection in *miR-1* transfected cells compared to the NC (1.70 fold decrease, n=4, $p < 0.0001$; Figure 1). A significant decrease in growth rate was also observed from 48 to 72 hours post-transfection in *miR-1* transfected cells compared to the NC (2.20 fold decrease, n=4, $p < 0.0001$) Similar results were observed in a replicate experiment. These results suggest *miR-1* reduces cell proliferation.

3.2 *MiR-1* Induces Apoptosis

To determine if *miR-1* induces apoptosis, *miR-1* and a scrambled precursor miR negative control (NC) were transfected into A5 cells, and the degree of apoptosis was measured at 24, 48, and 72 hours post-transfection using a Caspase-Glo 3/7 assay (Figure 2). A significant increase in apoptosis was observed at 48 and 72 hours post transfection in *miR-1* transfected cells compared to the NC (48 hour: 1.61 fold increase; n=3; $p < 0.0001$ and 72 hour: 2.07 fold increase; n=3; $p < 0.05$). These results suggest *miR-1* induces apoptosis, which, in part, may account for the observed decrease in cell proliferation at 72 hours post-transfection. Subsequent experiments did not show significant differences in apoptosis between *miR-1* and NC transfected cells at 48 and 72 hour post-transfection. Further experimentation must be performed to clarify the role of *miR-1* in apoptosis.

Effect of *miR-1* on Apoptosis

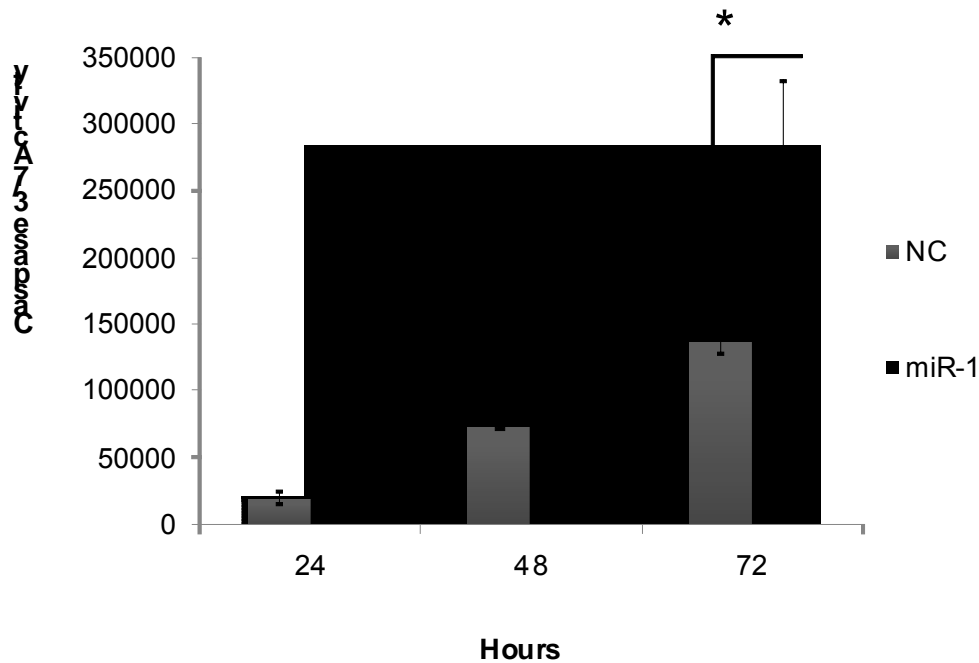


FIGURE 2. Apoptosis (~caspase 3/7 activity) was measured via a Caspase-Glo 3/7 assay at 24, 48, and 72 hours post-transfection in A5 cells transfected with *miR-1* (black) and a scrambled precursor miR negative control (gray, NC). Apoptosis was measured in triplicate. **, $p < 0.0001$. *, $p < 0.05$.

3.3 *MiR-1* Reduces Cell Migration

To determine if *miR-1* decreases cell migration, *miR-1* and a scrambled precursor miR negative control (NC) were transfected into A5 cells. At 60 hours post-transfection, the cells were placed were subject to a Transwell migration assay in which the cells were allowed to migrate through a fibronectin-coated membrane for 12 hours. Crystal violet staining was performed and the degree of migration was assessed using spectrophotometry (Figure 3). Cells transfected with *miR-1* experienced a 1.30 fold decrease in migration compared to cells transfected with a negative control ($n=3$, $p < 0.05$).

Effect of *miR-1* on Migration

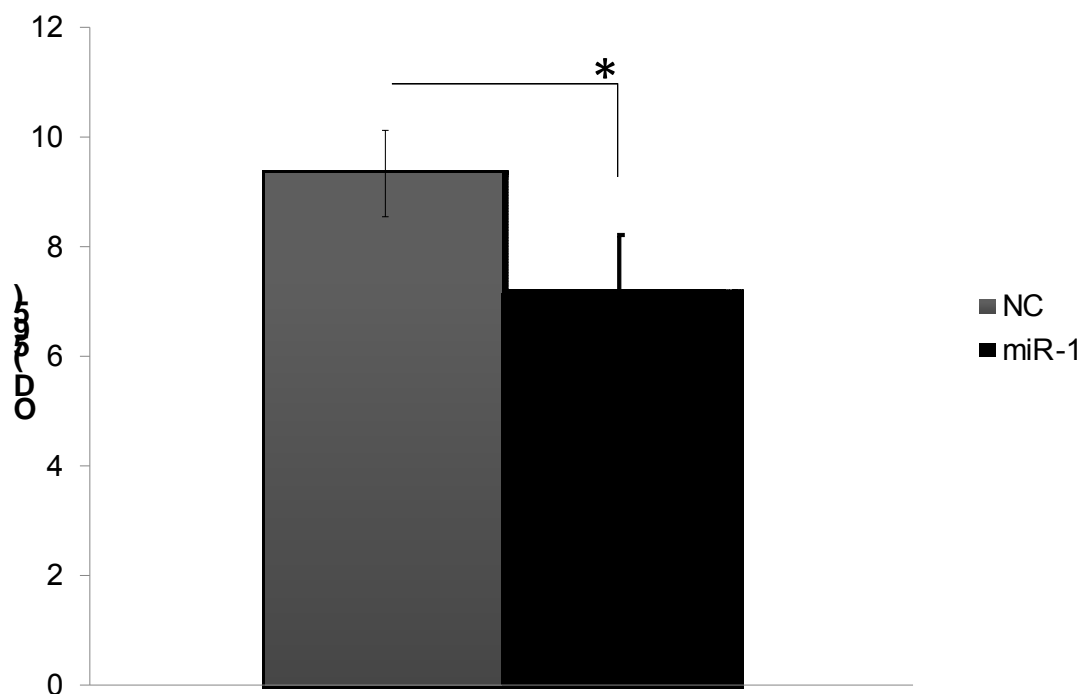


FIGURE 3. Migration (OD 595) was measured via a Transwell migration assay and subsequent crystal violet staining at 72 hours post-transfection in A5 cells transfected with *miR-1* (black) and those transfected with a scrambled precursor miR negative control (grey, NC). n=3, *, p < 0.05.

3.4 *MiR-1* Down-regulates Various Target Genes

Using the in silico predication programs, www.microRNA.org and www.targetscan.org, various predicted *miR-1* targets were identified based on complementary alignment of *miR-1* to the target gene's 3'UTR (Table 2). Initially, the genes *Ets1*, *Met*, *Bag4*, *Sp1*, *Taok1*, *Trp53*, and *Zfp148* were chosen because they had the highest number of *miR-1* binding sites in their 3'UTR. From this list of targets, *Ets1*, *Met*, and *Bag4* were chosen to perform expression studies based on the number of binding sites and literature review on their role in cellular processes. *Ets1* (E26 avian leukemia oncogene 1) is a transcription factor oncogene over-expressed in cutaneous

SCC thought to regulate genes important in apoptosis, cell proliferation, and migration [15,36,37,38,39,41,52]. *Met* encodes c-*Met*, which has been shown to be implicated in the progression of various cancers and has also been shown to be a target of *miR-1* in rhabdomyosarcoma. As a kinase, c-*Met* activates proteins via phosphorylation. [27,52,53]. *Bag4* (Bcl-2 associated athanogene 4) is an anti-apoptotic co-chaperone for Bcl-2 that has been shown to contribute to progression of breast, pancreatic, gastric, and ovarian cancers [54,55,56,57,58]. *Ptk9* is a known target of *miR-1* used as a positive control. *Ets1*, *Met*, and *Bag4* all have greater than one *miR-1* target site on their 3'UTR. Also, all have predicted target sites in *Homo sapiens*, and both *Ets1* and *Met* have one conserved target site between human and mice.

Potential *miR-1* Targets

Target	# Target Sites- <i>Mus musculus</i>	# Target Sites- <i>Homo sapiens</i>	# Conserved Target Sites	Fold Decrease mRNA Expression (48 HR)	Fold Decrease mRNA Expression (72 HR)	Fold Decrease Protein Expression (72 HR)
<i>Ets1</i>	3	2	1	1.1	1.71***	8.75
<i>Met</i>	2	2	1	2.45**	1.66**	n/a
<i>Bag4</i>	3	1	0	0.93	2.02	n/a
<i>Ptk9</i>	1	1	0	3.16***	1.98***	n/a

TABLE 2. Two *in silico* prediction programs, www.microRNA.org and www.targetscan.org were used to identify various target genes of *miR-1*, the number of *miR-1* target sites on their 3'UTR in *M. musculus* and *H. sapiens*, as well as the number of conserved target sites between the two species. *Ptk9* is a known *miR-1* target used as a positive control. *miR-1* and a scrambled precursor miR negative control (NC) were transfected into A5 cells, and the fold decrease in mRNA and protein expression between *miR-1* transfected and NC transfected cells was calculated after performing quantitative real time PCR and western blotting respectively. mRNA expression was measured in triplicate. Protein expression was measured in one sample. n=3, ***, p <0.002. **, p <0.02.

Effect of miR-1 on Potential Target Gene Expression

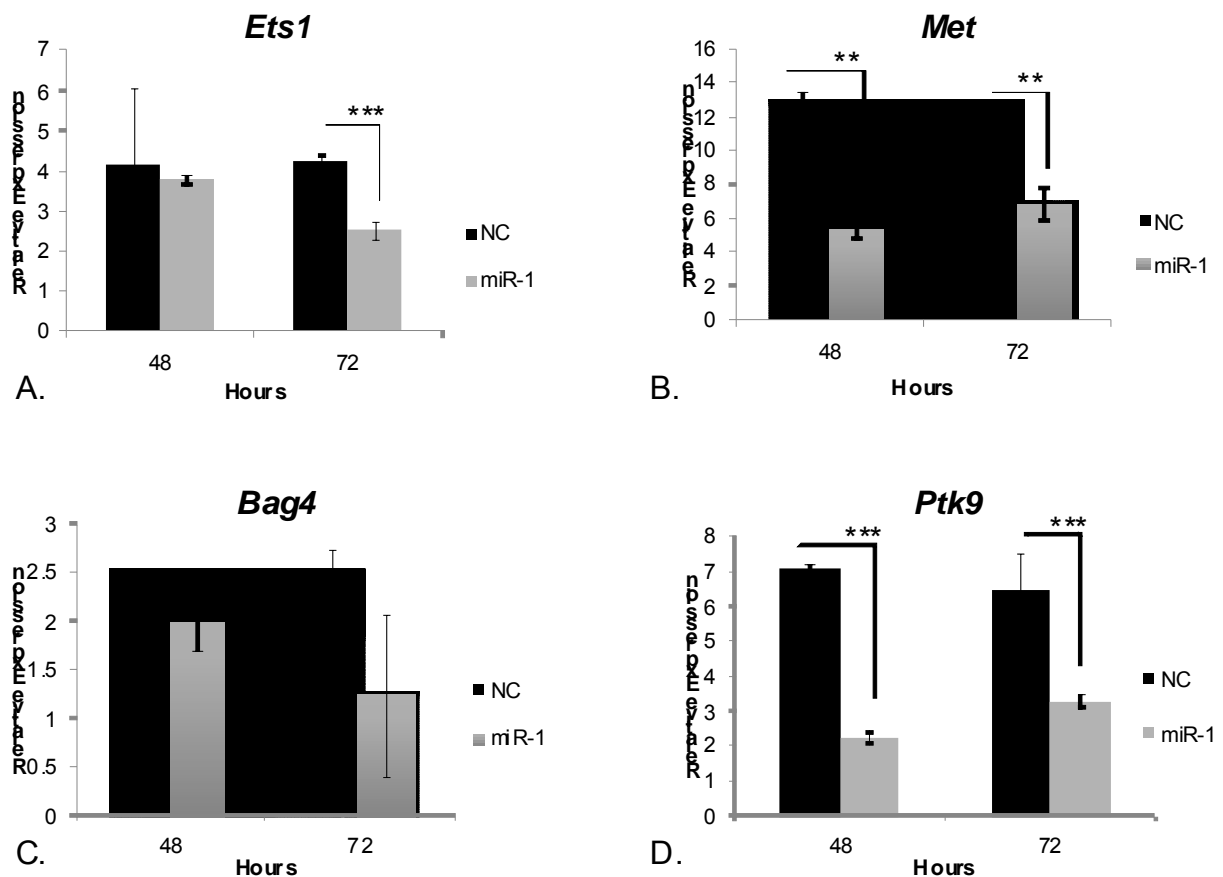


FIGURE 4. *Ets1* (A), *Met* (B), *Bag4* (C), and *Ptk9* (D) mRNA expression were measured via SYBR Green qPCR at 48 and 72 hours post-transfection in cells transfected with either a scrambled precursor miR negative control (NC; black) or *miR-1* (grey). n=3, ***, p < 0.002. **, p < 0.02.

Translational Repression of *Ets1* via *miR-1*

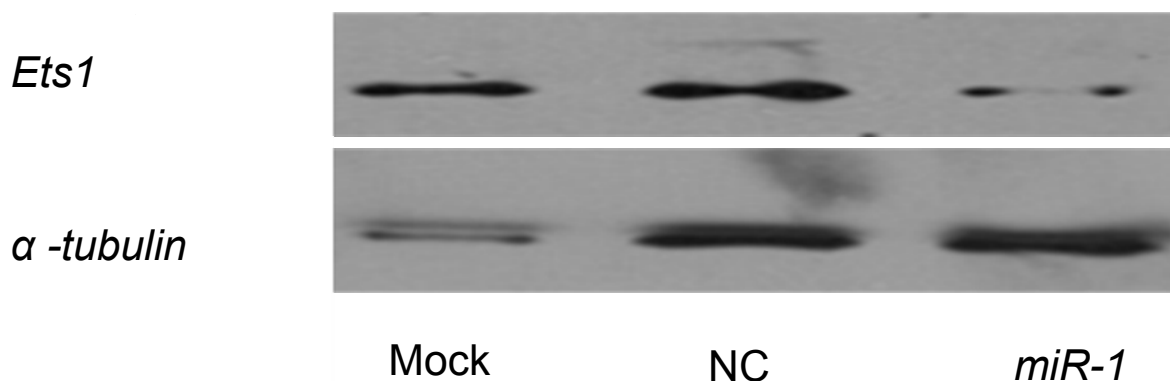


FIGURE 5. *Ets1* protein expression was measured via western blotting at 72 hours post-transfection. *Ets1* expression is decreased in A5 cells transfected with *miR-1* compared to cells transfected with no RNA (mock) or a negative control (NC),

Upon transfection of *miR-1* and a scrambled precursor miR negative control (NC) into A5 cells, the expression of the target genes was measured using quantitative real time PCR and western blotting (protein has currently only been measured for *Ets1*). A significant decrease in *Ets1* mRNA expression is observed at 72 hours post-transfection in cells transfected with *miR-1* compared to those transfected with the NC (Figure 4; n=3, p<0.002). Although significance levels could not be calculated because n=1, *Ets1* protein expression was also shown to be decreased dramatically at 72 hours post transfection in cells transfected with *miR-1* compared to those transfected with the NC (Figure 5). A significant decrease in *Met* mRNA expression is observed at both 48 and 72 hours post-transfection in cells transfected with *miR-1* compared to those transfected with the NC (Figure 4; n=3, p<0.02). *Bag4* mRNA expression was not significantly decreased by *miR-1*, but it could still be a target of translational repression. Finally, *Ptk9* mRNA expression is decreased at both 48 and 72 hours post-transfection in cells transfected with *miR-1* compared to those transfected with the NC (Figure 4; n=3, p<0.002). Based on inverse correlation of expression, these data suggests *Ets1* and *Met* are both potential targets of *miR-1* in cutaneous SCC.

3.5 *MiR-1* Regulates *Ets1* Expression via the 3'UTR

To determine if *miR-1* regulates *Ets1* via the 3' untranslated region (3'UTR), we created two luciferase constructs containing either the *Ets1* 3'UTR (Luc-*Ets1* 3'UTR) or the *Ets1* 3'UTR with mutated *miR-1* binding sites (Luc- *Ets1* 3'UTR-M). We performed site-directed mutagenesis to alter two base pairs in each of the *miR-1* binding sites in the *Ets1* 3'UTR. The mutations did not confer any more microRNA binding sites (Table 3). The constructs were co-transfected with either a precursor to *miR-1* or a scrambled precursor miR negative control (NC).

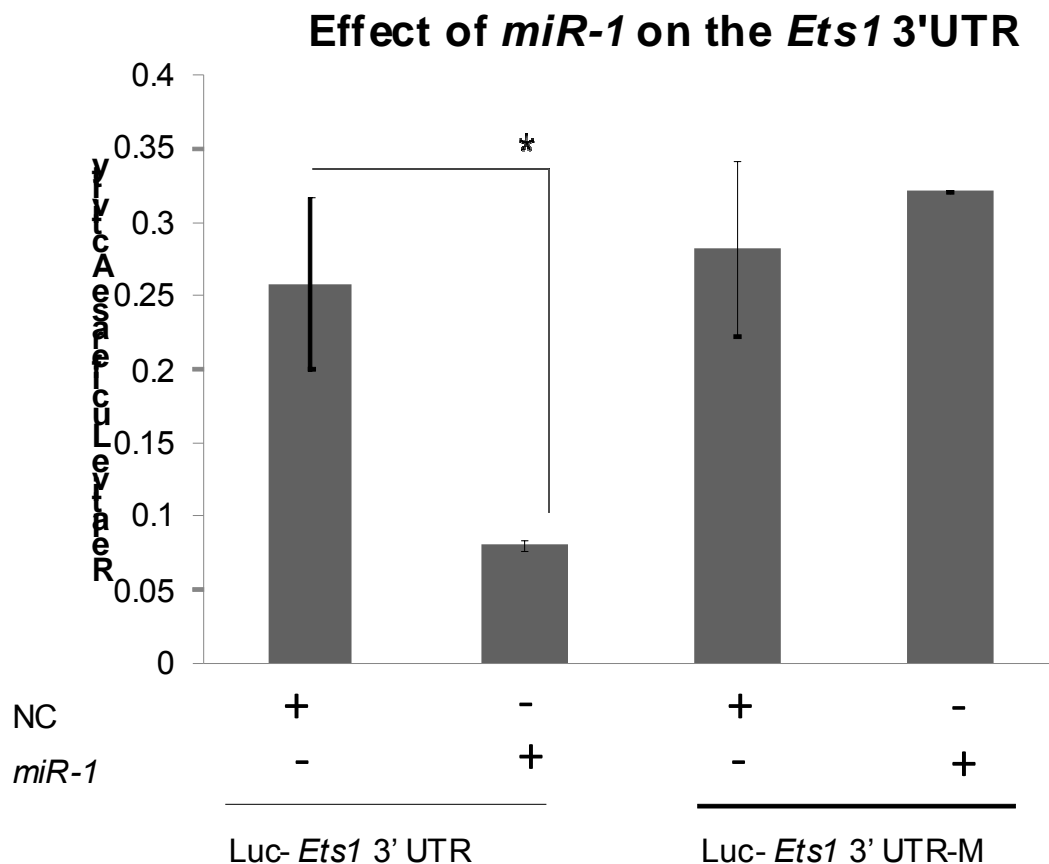


FIGURE 6. The *Ets1* 3'UTR and the *Ets1* 3'UTR with mutated *miR-1* binding sites (*Ets1* 3'UTR-M) were each cloned into a luciferase construct. The constructs were then co-transfected with either a negative control (NC) or *miR-1* and luciferase activity was measured. *Ets1* 3'UTR was created via site-directed mutagenesis. n=3 , *p < 0.007. This experiment was performed by Jessica Fleming.

A 3.2 fold decrease in luciferase expression was observed in cells co-transfected with Luc-*Ets1* 3'UTR and *miR-1* compared to those co-transfected with a NC, which suggests *miR-1* regulates *Ets1* through the *Ets1* 3'UTR (Figure 6; n=3, p < 0.007). No significant difference in luciferase expression was observed in cells co-transfected with Luc-*Ets1* 3'UTR-M and *miR-1* compared to those co-transfected with Luc-*Ets1* 3'UTR-M and a NC. This suggests *miR-1* regulates *Ets1* expression via direct binding to the predicted *miR-1* binding sites.

<i>miR-1</i> Binding Site in the <i>Ets1</i> 3'UTR	Site 1	Site 2	Site 3
NORMAL	5' ACUCC—CACA <u>U</u> UCCc 3'	5' ACAUUC C 3'	5' CUUACGUACA <u>U</u> UCC 3'
MUTATED	5' ACUCC—CACA <u>CU</u> Cc 3'	5' ACA <u>CU</u> CC 3'	5' CUUACGUAC <u>UC</u> UCC 3'

TABLE 3. Luc-*Ets1* 3'UTR-M was created via site-directed mutagenesis of the three *miR-1* binding sites. Two base pairs were mutated in each of the normal binding sites. The new, mutated binding sites did not confer new microRNA binding sites.

3.6 *Ets1* is Unable to Rescue the Effect of *miR-1* on Cell Proliferation

MiR-1, an *Ets1* promoter cyclomegalovirus construct lacking the 3'UTR (*Ets1*), and a scrambled precursor negative control (NC) were transfected into A5 cells to determine if *Ets1* is responsible for the increased cell proliferation in cells lacking *miR-1*. *Ets1* and *miR-1* (*Ets1* + *miR-1*) were also co-transfected into A5 cells. Cell proliferation was measured via an MTT assay at 24, 48, and 72 hours post-transfection. A significant increase in cell number was observed at 72 hours post-transfection in cells transfected with *Ets1* compared to cells transfected with either a NC, *Ets1*+ *miR-1*, or *miR-1*. [Figure 7; *Ets1*:NC, 1.81 fold increase; *Ets1*:(*Ets1* + *miR-1*), 2.44 fold increase; *Ets1*:*miR-1*, 2.04 fold increase; n=4; p<0.0001]. A significant increase in growth rate was also observed from 48 to 72 hours post-transfection in cells transfected with *Ets1* compared to cells transfected with either a NC, *Ets1*+*miR-1*, or *miR-1*. [Figure 7; *Ets1*:NC, 1.88 fold increase; *Ets1*:(*Ets1* + *miR-1*), 3.11 fold increase; *Ets1*:*miR-1*, 2.75 fold increase; n=4; p<0.0001].

Although a significant difference in cell number was not observed for cells transfected with a *miR-1* or *Ets1*+*miR-1* compared to cells transfected with a negative control at 72 hours post-transfection, a significant decrease in growth rate was observed for cells transfected with a *miR-1* or *Ets1*+*miR-1* compared to cells transfected with a NC from 48 to 72 hours post-transfection. [Figure 7; (*Ets1*+ *miR-1*): NC, 1.73 fold decrease, n=4, p<0.02; *miR-1*: NC, 1.50 fold decrease, n=4, p <0.01]. It appears that *Ets1* acts to increase cell proliferation and growth in the absence of *miR-1*, but cannot rescue the affects of decreased proliferation growth rate induced by *miR-1*. Yet, as confirmed in an earlier MTT assay, the presence of *miR-1* still decreases the growth

rate of A5 cells from 48 to 72 hours post-transfection. Overall, these results suggest *miR-1* may affect proliferation and growth rate by down-regulating target proteins other than *Ets1*.

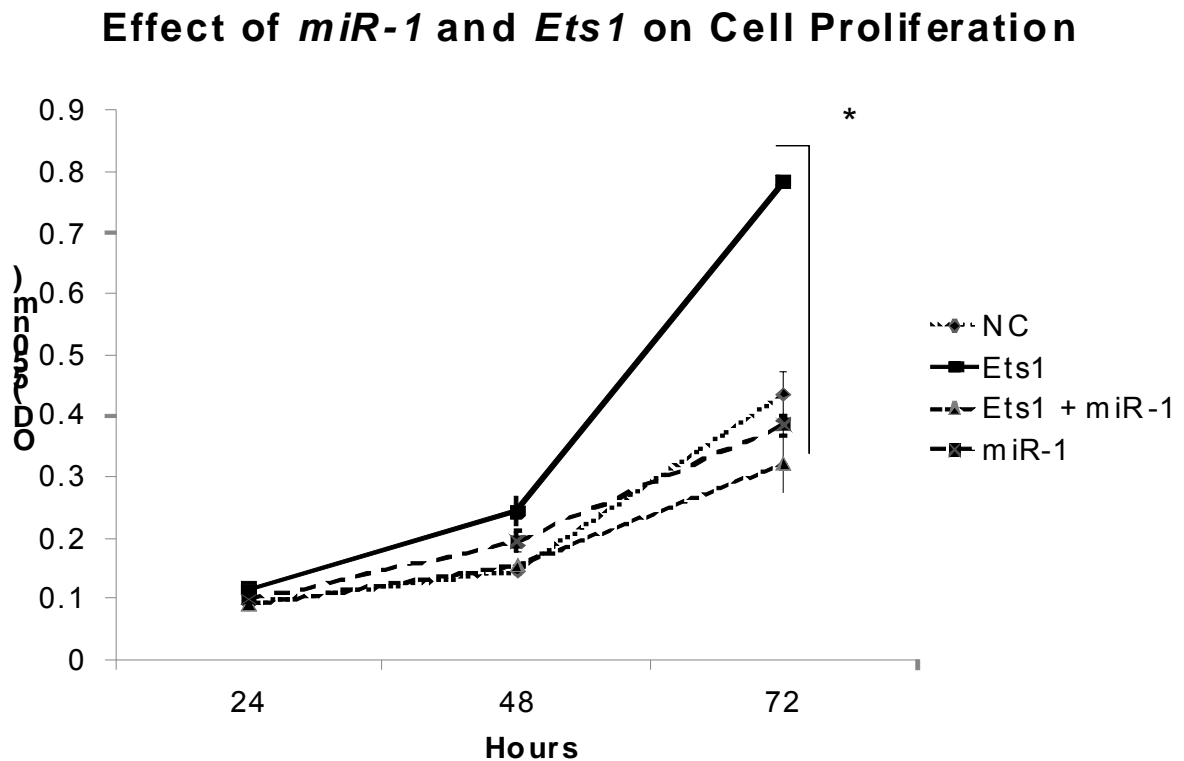
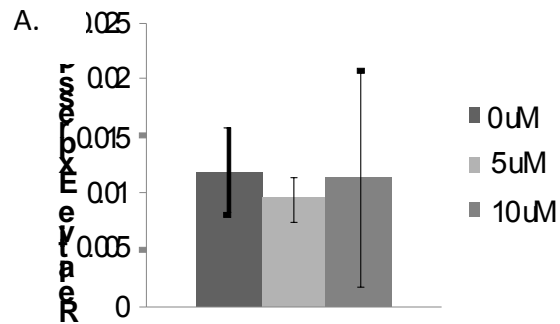


FIGURE 7. *MiR-1* (dashed square), an *Ets1* PCM construct lacking the 3'UTR (square, *Ets1*), and a scrambled precursor miR negative control (diamond, NC) were transfected into A5 cells. *Ets1* and *miR-1* (triangle, *Ets1+miR-1*) were also co-transfected into A5 cells. Cell proliferation was measured via an MTT assay at 24, 48, and 72 hours post-transfection. Proliferation at each time point was measured in quadruplicate. *, $p < 0.0001$.

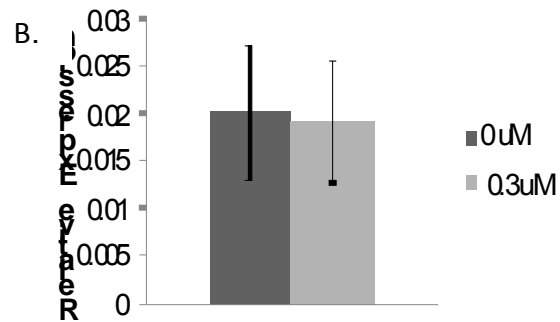
3.7 Methylation is not Responsible for Down-regulating *miR-1*

To determine if methylation was responsible for the differential *miR-1* expression in tumor cell lines, we treated A5 cells with 5-Azacytidine (5-AzaC), trichostatin A (TSA), or a combination of both (5-AzaC + TSA). RNA was isolated from cells treated with 5-AzaC at 48 hours post-transfection and cells treated with TSA or 5-AzaC + TSA at 24 hours post-transfection. *MiR-1* expression was measured via qPCR, and no significant change in expression was seen in treated versus non-treated cells (Figure 8).

Effect of 5-AzaC on *miR-1*



Effect of TSA on *miR-1*



Effect of 5-AzaC+ TSA on *miR-1*

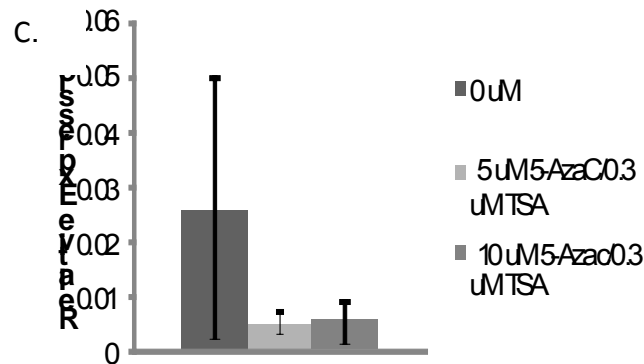


FIGURE 8: Relative *miR-1* expression was measured via Taqman Real Time PCR in A5 SCC cells after treatment with either 5-azaC, TSA, or a combination of the two drugs. Treatments were performed with 5-azaC for 48 hours at 5 uM and 10 uM concentrations (A), TSA for 24 hours at 0.3 uM concentration (B), and a combination of the two drugs with 5-azaC for 48 hours at 5 uM and 10 uM concentrations and TSA for 24 hours at 0.3 uM concentration (C). *MIR-1* expression was not significantly different between treated and untreated cells. *These experiments were performed by Jessica Fleming.*

3.8 Chromatin Silencing is Not Responsible for Down-regulating *miR-1*

Because the mouse promoter for *miR-1* contains no CpG islands, segments of DNA where methylation is likely, we wanted to determine if histone silencing might be occurring in the absence of DNA methylation. A chromatin immunoprecipitation (ChIP) assay was performed using DNA/protein complexes isolated from A5 and C5N cell lines. Semi-quantitative analysis of the ChIP product revealed histone silencing of the *miR-1* promoter, but with no significant difference in A5 cells compared to C5N (Figure 9).

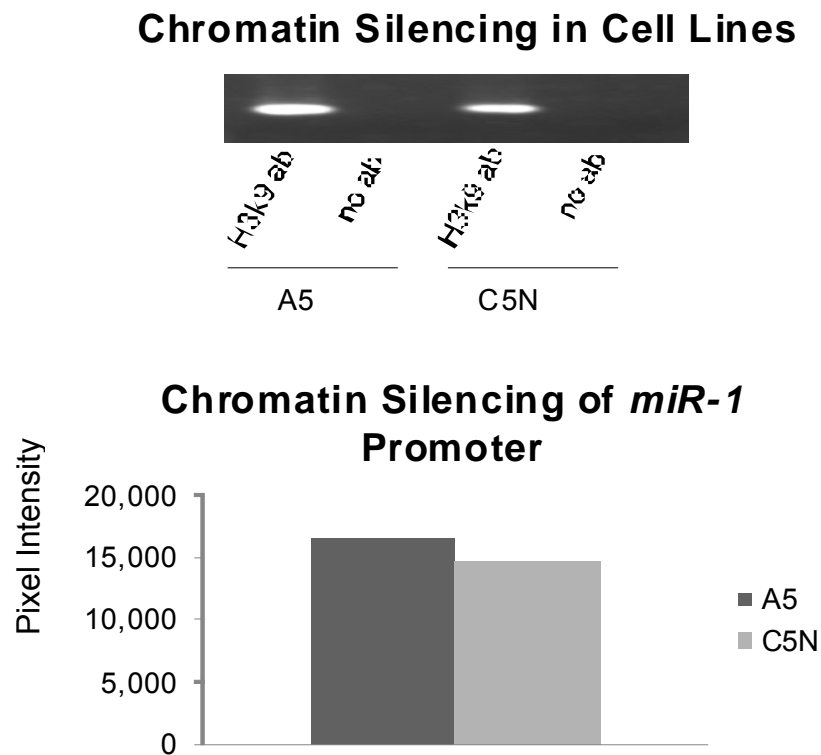


FIGURE 9. A ChIP assay was performed on A5 and C5N cells to detect chromatin-silencing in the *miR-1* promoter region. A5 and C5N chromatin/DNA complexes were isolated via H3K9 specific antibody (ab) from which the *miR-1* promoter region was amplified through PCR (A). Semi-quantification of the ChIP assay PCR product was performed to determine the relative degree to which A5 and C5N cell lines are chromatin-silenced. To amplify the *miR-1* promoter region, a PCR was performed with 20 ng of the A5 and C5N DNA retrieved after the ChIP assay, and then the PCR product band intensities were measured via computer analysis. Minimal difference in chromatin silencing between A5 and C5N cell lines was observed (B).

CHAPTER 4

DISCUSSION

We hypothesized that *miR-1* acts as a tumor suppressor in cutaneous squamous cell carcinoma by affecting various phenotypes including cell proliferation, apoptosis, and migration and that it also down-regulates the transcription factor oncogene, *Ets1*. Further, we tested for methylation and chromatin silencing in the *miR-1* promoter region to determine the mechanism for down-regulation of *miR-1* in SCC. In summary, we had three aims to our study:

1. Determine the phenotypes associated with *miR-1* in SCC.
2. Verify *Ets1* was a direct target of *miR-1*, and that *miR-1* down-regulated *Ets1* via binding to the 3'UTR.
3. Determine the mechanism of down-regulation of *miR-1* in SCC.

4.1 *MiR-1* Affects Tumor Suppressor Phenotypes

From our results, *miR-1* transfection results in a decrease in cell number and migration at 72 hours as well as a dramatic decrease in growth rate between 48 and 72 hours post-transfection in cells transfected with *miR-1* compared to those transfected with a scrambled precursor miR negative control (NC). Furthermore, apoptosis was also significantly increased at 48 and 72 hours post-transfection in cells transfected with *miR-1* compared to those transfected with the NC. This suggests that apoptosis, in part, may be decreasing cell proliferation by inducing apoptosis, and other studies have shown *miR-1* to act similarly in lung, liver, and skeletal muscle cancers [25,26,27].

Cell death may be responsible for the decreased number of cells; however, it is also possible that *miR-1* may be regulating expression of proteins important in cell cycle regulation. In this case, cells may not necessarily be undergoing apoptosis, but dividing less rapidly. Both apoptosis and cell cycle inhibition could be inhibited by *miR-1*. To determine if cell cycle inhibition contributes to the decrease in proliferation, it will be necessary to transfect cells with *miR-1* and perform flow cytometry to determine what stage of the cell cycle (e.g. G₀, G₁, G₂, S, or M) the majority of cells are in compared to the NC.

4.2 *Ets1* is a target of *miR-1*

After identifying *Ets1* and *Met* as potential targets of *miR-1* using the *in silico* prediction programs, as well as literature review, expression studies revealed that both genes are down-regulated at the mRNA level upon introduction of exogenous *miR-1*. Further, *Ets1* is significantly down-regulated at the protein level upon introduction of exogenous *miR-1*, which coincides with a commonly accepted mechanism of microRNA regulation via translational inhibition [11].

Not only do we confirm *miR-1* down-regulates *Ets1*, but we have shown it does so by directly binding to the 3'UTR. This has major implications as it provides an approach for potential SCC therapies using the *miR-1* pathway. Although it appears that *Ets1* does not affect proliferation, if we can show preferentially knocking down *Ets1* induces other phenotypes (potentially increased apoptosis, and decreased migration) as *miR-1*, then therapies could be focused not necessarily on reintroducing *miR-1* but by introducing an *Ets1* siRNA. Although microRNA therapy has many advantages,

microRNAs have the capability to down-regulate many genes which could lead to deleterious side effects as not all the genes are known or functionally understood. A siRNA only down-regulates one specific gene, which would allow a therapy to have fewer undesired effects.

4.3 *Ets1* Increases Cell Proliferation but Cannot Rescue the Effects of *MiR-1*

Upon co-transfection of *Ets1* and *miR-1* into A5 cells, *Ets1* could not simultaneously increase proliferation that was suppressed by *miR-1*; however, in isolation it caused a dramatic increase in cell proliferation at 72 hours. From this, we can conclude *Ets1* does act to increase cell proliferation. This study suggests that *miR-1* acts to decrease cell proliferation, but not by down-regulation of *Ets1* because exogenous *Ets1* did not rescue its effects. The *Ets1* construct did not contain a 3'UTR, so *miR-1* would be unable to down-regulate exogenous *Ets1*, which rules out the conclusion that the exogenous *Ets1* was degraded. This can be verified in future studies via western analyses of transfected cells *Ets1*. However, it is possible and likely that *miR-1* regulates various targets responsible for decreasing proliferation; therefore, the amount of transfected exogenous *Ets1* was not enough to rescue the effects resulting from down-regulation of other target genes. Rescuing the effects of *miR-1* may require re-introduction of various target genes.

Future experimentation will be performed using different dosage amounts of *miR-1* and *Ets1* in transfections to confirm that neither one is over-expressed to a degree that will mask the effects of the other. A simple way to validate this would be to compare protein expression in co-transfected cells with *miR-1* transfected cells in an

attempt to identify the dosage of *Ets1* required to restore endogenous levels of *Ets1* to normal levels. Finally, in future studies, co-transfection of *Ets1* and *miR-1* will be repeated in attempt to determine if *Ets1* rescues the effect of induced apoptosis by *miR-1*.

Another approach to identifying the phenotypes associated with *Ets1* is to utilize an *Ets1* siRNA. The siRNA will preferentially knock down *Ets1* expression. After transfecting this siRNA into the A5 cell line, phenotypes such as proliferation, apoptosis, migration, etc. can be assessed using similar experimentation as those previously used to determine phenotypes associated with *miR-1*. We have recently been attempting to knock down *Ets1* with a siRNA; however, no knock-down is consistently observed. This may be because we have not yet found the optimal concentration of siRNA necessary for sufficient knockdown. We are continuing to trouble shoot and optimize experimentation.

4.4 Down-regulation of *MiR-1* in Cutaneous SCC

Our studies suggest that *miR-1* is not down-regulated via chromatin silencing or methylation; however, there are other forms of transcriptional regulation to be considered.

First, we can consider other forms of regulation of the *miR-1* promoter region. There is the possibility that SNPs arise in the *miR-1* promoter region of susceptible mice and SCC cells, which could possibly cause differential transcription factor binding. Previously, we sequenced the *miR-1* promoter region in susceptible and resistant mice, and submitted the sequencing to the Transcriptional Element Search Software in order

to investigate potential differential transcription factor binding between *miR-1* promoter region of the two strains of mice. The studies showed that the transcription factor, *Gal4*, may bind to the *miR-1* promoter of resistant mice but not susceptible. Further, a study in lung cancer showed that *miR-1* is regulated by the tumor suppressing C/EBP α transcription factor [25]. Future studies can focus on performing expression studies on these various transcription factors in order to determine if altered levels may correlate with the *miR-1* down-regulation. If correlation exists, we can move to conduct functional studies such as reintroducing potential tumor suppressing transcription factors into SCC cell lines in order to rescue *miR-1* expression.

Similar to the approach of investigating *miR-1* regulation via the promoter region, we could also consider *miR-1* enhancer regions. *In silico* prediction programs such as <http://enhancer.lbl.gov/> allows us to predict enhancer binding domains, and these domains could be sequenced in search of SNPs and differential binding of enhancer proteins. Studies suggest that alterations in distant enhancer regulatory regions could possibly contribute to increased susceptibility to carcinogenesis [59].

4.5 *MicroRNA-1* and *Ets1* as Potential Diagnostic Bio-markers and Therapeutics

Our studies indicate that *miR-1* may act as a SCC tumor suppressor, in part, via down-regulating *Ets1* which could lead to potential future clinical applications. *MiR-1* levels could possibly be used as a biomarker. Studies indicate that some microRNAs in lung, breast, and ovarian cancer are found in circulation, in which case they could potentially serve as a prognostic or diagnostic factor dependent on a relatively simple procedure of measuring their expression in blood [60,61,62]. Down-regulation of *miR-1*

or up-regulation of *Ets1* could correlate to the risk of eventually being diagnosed with SCC or serve as a prognostic factor for determining the severity of the disease.

MiR-1 could also be used as a future SCC therapy. Current treatment for SCC is most commonly surgical removal which is often painful, costly, and even increases the risk of cosmetic disfigurement. If SCC does in fact metastasize, chemotherapeutic approaches to treatment are often associated with unwanted side effects and are not cell specific. MicroRNAs are enticing therapies as current research is being focused on developing adenoviruses that can be tissue and cancer cell specific [12]. Also, an argument can be made in advocating for the use of microRNAs over siRNAs as therapies in cancer because microRNAs have the potential to target many genes. If one microRNA targets multiple genes that are involved in the same mechanism of tumorigenesis, then there should hypothetically be a greater effect. Finally, minimally invasive injection or intranasal administration of these microRNA therapeutics are currently being investigated, which could eliminate the pain and discomfort associated with cosmetic removal [12].

In conclusion, great advancements have been made in microRNA research over the past two decades, and they continue to do so. MicroRNAs have the potential to one day be effective, efficient treatment options for various cancers, including cutaneous SCC. Determining important microRNAs and their targets such as *miR-1* and *Ets1* in SCC could help enhance our control of this increasingly common disease and alleviate morbidity, and even in rare cases, mortality.

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